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Androgen Stimulates Transcription and Replication of Xenotropic Murine Leukemia Virus-Related Virus[▽]

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Xenotropic murine leukemia virus-related virus (XMRV) is a gammaretrovirus originally identified in a subset of prostate cancer patients. Because androgens stimulate prostate tumors and some retroviruses, we investigated the effects of dihydrotestosterone (DHT) on XMRV transcription and replication. Transcription from the XMRV U3 region was stimulated up to 2-fold by DHT, but only in cells containing a functional androgen receptor. Mutations in the glucocorticoid response element (GRE) of XMRV impaired basal transcription and androgen responsiveness. Furthermore, DHT stimulated XMRV replication 3-fold, whereas androgen inhibitors (casodex and flutamide) suppressed viral growth up to 3-fold. Findings suggest that integration of the XMRV long terminal repeat (LTR) into host DNA could impart androgen stimulation on cellular genes.

Xenotropic murine leukemia virus-related virus (XMRV) is an infectious gammaretrovirus identified in tumor-bearing prostates of men homozygous for a reduced-activity variant of the antiviral enzyme RNase L (8, 19). More recently, XMRV has also been detected in 67% of chronic fatigue syndrome patients and 3.7% of normal-health control individuals (13). A genome-wide analysis of XMRV integration sites in a prostate cancer cell line and in human prostate cancer tissues revealed a preference for transcription start sites, CpG islands, DNase-hypersensitive sites, and high-gene-density regions (10). Furthermore, XMRV integration sites in human prostate DNA showed a preference for cancer-related genes and breakpoints, common fragile sites, and microRNA genes. Those findings suggested that XMRV integration might cause dysregulation of select host genes possibly contributing to oncogenesis. In addition, multiple XMRV proviruses were recently identified in human 22Rv1 prostate carcinoma cells, suggesting a role for viral integration in carcinogenesis (11).

Transcription of the XMRV genome is mediated by elements in the U3 region of the 5′ long terminal repeat (LTR), a 390-nucleotide (nt) segment that includes the core promoter and enhancers (Fig. 1A). Many retroviruses contain a glucocorticoid response element(s) (GRE) in the U3 region, including XMRV and other gammaretroviruses, such as Moloney murine leukemia virus (Mo-MLV) and Friend murine leukemia virus (F-MLV) as well as the betaretrovirus mouse mammary tumor virus (MMTV) (2, 3, 17, 19) (Fig. 1B). Viral GREs are stimulated in response to various steroids, including glucocorticoids, mineralocorticoids, progesterone, and androgen (1, 4, 5, 7, 14). Viral GREs often have homology to the classical androgen response element (ARE), a binding site for dimers of the androgen receptor (AR) that consists of an inverted 6-bp repeat separated by a 3-bp spacer (Fig. 1B) (16). There is homology between these viral GREs and AREs in some mammalian genes. For example, the first inverted repeat, comprising positions −7 to −2 (AGAACA), in the XMRV GRE is identical to the prostate-specific antigen (PSA) ARE1 (6).

To determine if the XMRV U3 region of the 5′ LTR contains a functional ARE, experiments were performed with the human prostate cancer cell line LNCaP, which expresses a functional androgen receptor (12) (Fig. 1C). The reporter plasmid pGL4.26-XMRV_U3 was constructed by digesting the pGL4.26 vector containing firefly luciferase cDNA (Promega, Madison, WI) and XMRV clone Vp62-pcDNA3.1(−) (GenBank accession no. EF185282) (8) with NheI and HindIII and inserting nt 7748 to the end of XMRV into pGL4.26. This segment of the XMRV genome includes all of the U3 region (except for 28 nt at the 5′ end) and the R region. Two mutations were generated in the putative ARE: (i) an A-to-G transition mutation at position −2 (AGAACA to AGAACG [the mutated position is underlined]) in the GRE (3) and (ii) a deletion of the 5′ half of the GRE (ΔAGAACA) (Fig. 1B). A QuikChange XL site-directed-mutagenesis kit from Stratagene (La Jolla, CA) was used to generate the point mutation with a pair of primers containing sequences producing an A7913-to-G mutation, and the deletion mutation was generated with primers, consisting of a deletion of the sequence AGAACA from nt 7908 to nt 7913. All mutations were confirmed by sequencing. Cells in 12-well plates cultured in hormone-depleted medium (phenol red-free RPMI medium 1640 plus 2% charcoal stripped fetal bovine serum [FBS]) (Invitrogen, Carlsbad, CA) were cotransfected using Lipofectamine 2000 (Invitrogen) with 1.0 μg of wild-type (WT) or mutant pGL4.26-XMRV_U3 and 0.1 μg of pRL-PK (encoding *Renilla* luciferase) for 24 h, followed by treatment of cells with 4,5a-dihydrotestosterone (DHT) (Sigma-Aldrich, St. Louis, MO) in fresh hormone-depleted medium. Cells were lysed after 24 h, and luciferase assays were performed with a dual-luciferase reporter assay system kit (Promega, Madison, WI). Firefly luciferase activity from pGL4.26-XMRV_U3 was normalized to *Renilla* luciferase activity and expressed as relative light units (RLU). The results

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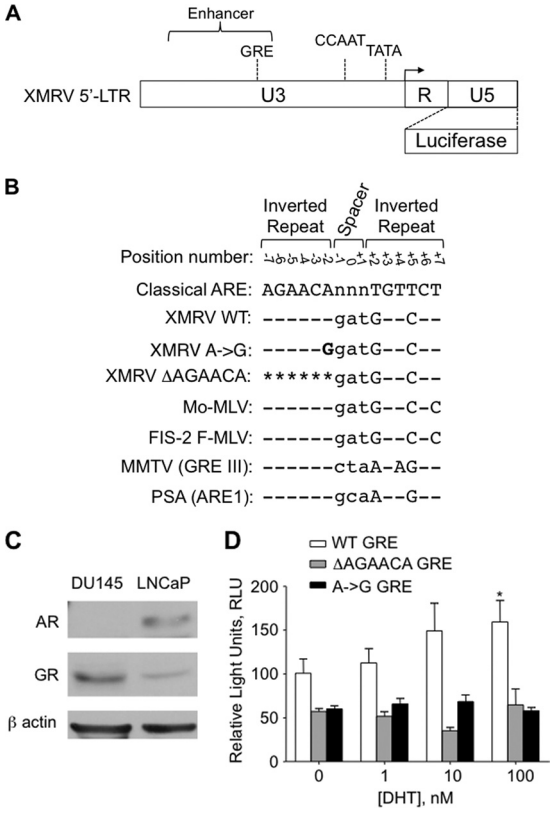

FIG. 1. Functional analysis of the XMRV GRE in LNCaP cells. (A) Map of the XMRV 5′ LTR and the promoter-luciferase construct. (B) Comparison of classical ARE with viral GREs, mutant XMRV GREs, and human PSA ARE1 (* indicates deleted nucleotides). (C) Western blot analysis for the androgen receptor (AR) and the glucocorticoid receptor (GR) in DU145 and LNCaP cells. Cell extracts (60 μg) were used, and the blot was probed with rabbit anti-androgen receptor antibody, followed by reprobing with rabbit anti-glucocorticoid receptor antibody (both from Santa Cruz, Inc.) after stripping. Antibody against β actin (Sigma-Aldrich) was used. (D) LNCaP cells were transfected with either wild type (WT) or mutant XMRV U3 (as indicated) fused with firefly luciferase cDNA in combination with *Renilla* luciferase cDNA for normalization of data. Relative light units (RLU) were determined by dividing firefly luciferase activity levels by *Renilla* luciferase activity levels after culturing in the absence or presence of dihydrotestosterone (DHT) for 24 h in hormone-depleted medium with 2% charcoal-stripped FBS. Experiments were reproduced two times, analyses were performed in triplicate, and standard deviations (SD) were calculated. *, P values of <0.05 as determined by a two-tailed, paired Student t test.

obtained using LNCaP cells showed that the transcriptional activity induced from WT U3 by 100 nM DHT was 158% ± 24% of the control (untreated) activity level and that the mutated U3 regions had reduced basal activities (to 56 to 60% of the WT U3 activity level) and were unresponsive to DHT (Fig. 1D).

Mo-MLV U3 contains a sequence that is highly similar to the GRE in XMRV U3 except for a 1-nt difference (Fig. 1B). To compare the U3 regions of XMRV with those of Mo-MLV, an additional promoter construct was made. Mo-MLV U3 (nt 7846 to 8332) was prepared by PCR from plasmid p63-2 of Mo-MLV (a gift from H. Fan, University of California, Irvine, CA) (9) and was inserted into pGL4.26 after digestion with NheI and HindIII. The relative activities of the XMRV and Mo-MLV U3 regions in human prostate cancer cell lines that express (LNCaP) or lack (DU145) the androgen receptor (Fig. 1C) (12) were directly compared (Fig. 2A and B, respectively) in the absence or presence of DHT, as determined by luciferase activity. In the LNCaP cells, DHT stimulated transcription from both the XMRV U3 and the Mo-MLV U3 regions about 2.0- and 1.8-fold, respectively (Fig. 2A). However, XMRV U3 was 1.6- and 1.8-fold more active than the Mo-MLV promoter in the absence and presence of DHT, respectively. In contrast, neither XMRV U3 nor Mo-MLV U3 was stimulated by DHT in the DU145 cells, which lack the androgen receptor (Fig. 2B). These findings are consistent with our prior observation that XMRV replicates to 4-fold-higher levels in LNCaP cells than in DU145 cells (8). To compare the androgen effect on XMRV transcription with that of another steroid hormone, DU145 and LNCaP cells which have a glucocorticoid receptor (Fig. 1C) were plated in hormone-depleted medium and transfected with pGL4. 26-XMRV-U3 for 24 h prior to treatment with the glucocorticoid dexamethasone for 24 h. Dexamethasone increased transcription from the XMRV U3 region in LNCaP and DU145 cells 1.3- and 1.5-fold, respectively (Fig. 2C).

To investigate the effect of androgen on viral replication, LNCaP cells were cultured in triplicate in 24-well plates in hormone-depleted medium for 2 days, until cells were nearly confluent, to minimize the effect of DHT on cell numbers. Cells were infected with XMRV in serum- and phenol red-free RPMI with 8 μg/ml polybrene (8). After 3 h of incubation, cells were washed once with phosphate-buffered saline (PBS) and

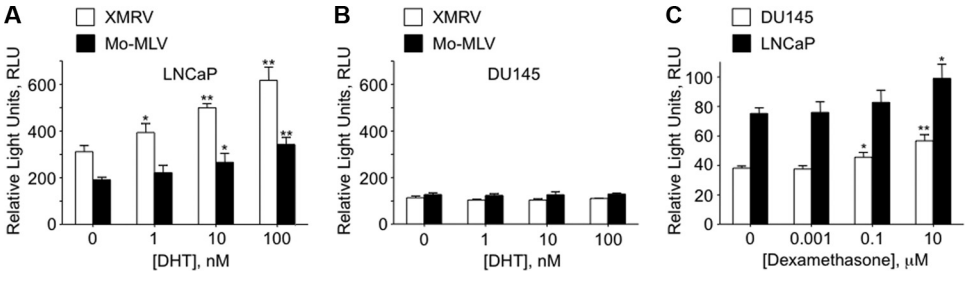

FIG. 2. Androgen stimulates transcription from the XMRV and Mo-MLV U3 regions in LNCaP (A) but not in DU145 (B) cells, and dexamethasone stimulates transcription of XMRV-U3 in both cell types (C). Cells were transfected with either XMRV U3 or Mo-MLV U3, each fused to firefly luciferase cDNA. DHT (A and B) and dexamethasone (C) treatments were for 24 h at the concentration indicated (x axis) in hormone-depleted medium with 2% charcoal-stripped FBS. Numbers of RLU were determined by normalization to the level for *Renilla* luciferase activity expressed from plasmid pRL. Experiments were reproduced two times. Error bars represent SD. *, P values of <0.05, and **, P values of <0.01 as determined by a two-tailed, paired Student t test.

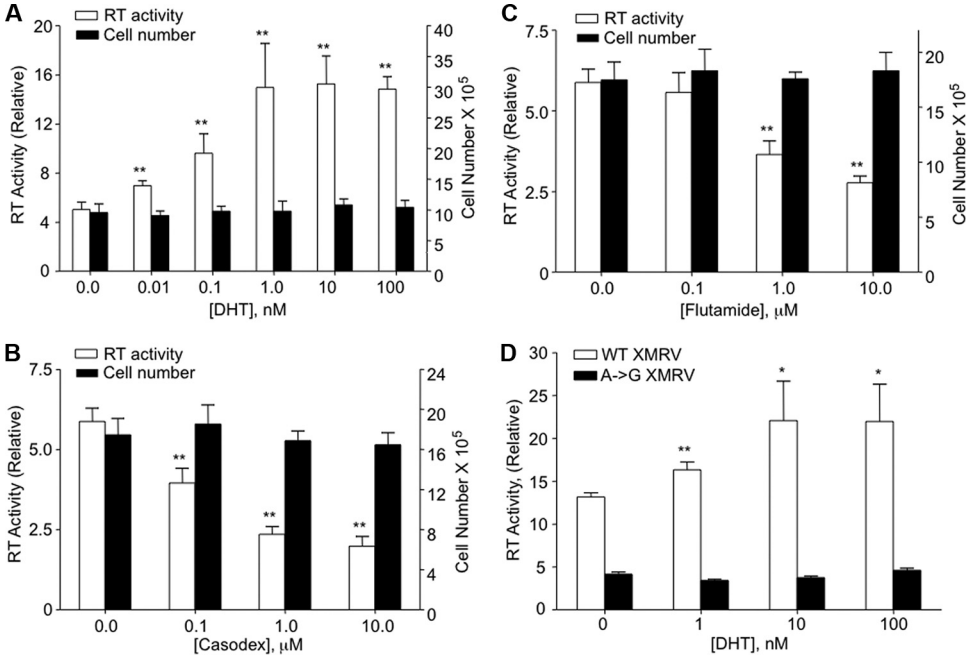

FIG. 3. XMRV replication is stimulated by androgen and suppressed by antiandrogens. LNCaP cells were cultured in 24-well plates with RPMI medium plus 10% FBS for 2 days, until cells were fully confluent. Cells were infected with XMRV in the presence of 8 μg polybrene/ml for 3 h, followed by washing of the cells with PBS and treatment with DHT (0.01 to 100 nM) in hormone-depleted medium for 2 days (A) or casodex (0.1 to 10 μM) (B) or flutamide (0.1 to 10 μM) (B) in RPMI medium plus 10% FBS for 4 days. (D) Comparison of effects of DHT on acute infections with WT XMRV and A-to-G GRE mutant XMRV in LNCaP cells. Experiments were done as described for panel A, except infections were for 3 days. Experiments were repeated two or three times, and each individual experiment was done in triplicate. RT activities were determined with conditioned cell medium. The cell numbers shown are per well. Error bars represent SD. *, P values of <0.05, and **, P values of <0.01 as determined by a two-tailed, paired Student t test.

treated with DHT in fresh hormone-depleted medium for 2 days. Cell numbers were determined, and conditioned cell medium was collected for reverse transcriptase (RT) assays performed with poly(rA) as a template and oligo(dT) as a primer (18). The relative RT activities of radiolabeled reaction products were determined with a Storm 840 phosphorimager (Sunnyvale, CA). DHT treatment stimulated XMRV replication to 304% ± 45% of the control level (Fig. 3A). To verify androgen regulation of XMRV replication, the LNCaP cells acutely infected with XMRV in regular RPMI medium containing 10% FBS were treated with different concentrations of an antiandrogen, either casodex or flutamide (Fig. 3B and C, respectively). The antiandrogens (at 10 μM) inhibited viral replication as measured by RT activity to 33.6% ± 5.2% (casodex) and 49.7% ± 3.6% (flutamide) of the control level. In comparison, DHT slightly increased cell numbers (111% ± 10% of the control level), while casodex and flutamide had no effect on cell numbers. In addition to these acute-infection experiments, viral expression of RT activity in LNCaP cells chronically infected with XMRV was stimulated by DHT more than 3-fold and similarly inhibited by casodex or flutamide (data not shown). Virus replication in DU145 cells was not affected by DHT, due to the absence of the androgen receptor in these cells (data not shown).

To determine if the XMRV GRE/ARE imparts androgen responsiveness on intact virus, we mutated A to G at position −2 (Fig. 1B and 3D) in plasmid encoding full-length XMRV strain VP62. WT and mutant viral stocks were prepared in LNCaP cells as described previously (8). LNCaP cells were infected with either WT or mutant XMRV with polybrene in hormone-depleted medium and cultured for 3 days in the absence or presence of DHT. Treatment with DHT stimulated WT XMRV replication to 167% ± 33% of the control level, as determined by measurement of RT activity in the conditioned medium. However, DHT had no effect on the replication of XMRV with the single-base mutation (A to G) at position −2 in the GRE/ARE (Fig. 3D).

Our findings show the presence of a functional androgen response element (ARE) in the U3 region of the XMRV LTR. The XMRV ARE sequence (AGAACAGATGGTCCT) is conserved among different XMRV strains (19). Inhibition of XMRV replication by antiandrogens suggests that viral growth could also be suppressed in XMRV-infected prostate cancer patients during androgen ablation therapy. Recently, we reported that XMRV integration sites in human prostate tumors include many cancer-related genes (8, 10). XMRV has a strong preference for integrating near transcriptional start sites, possibly imposing androgen responsiveness on host genes. XMRV has been observed in both prostatic stromal (19) and epithelial carcinoma (11, 15) cells. Therefore, XMRV integration into stromal and epithelial cells could cause androgen stimulation of proinflammatory genes and proto-oncogenes leading to cancer. In addition, because the androgen receptor is expressed in prostatic cells, these findings could also help to explain why XMRV infections localize to the prostate.

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